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A Role for CTCF and Cohesin in Subtelomere Chromatin Organization, TERRA Transcription, and Telomere End Protection

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Review timeline:

Submission date:	08 May 2012
Editorial Decision:	29 May 2012
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Accepted:	29 August 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 May 2012

Thank you again for submitting your manuscript for consideration by The EMBO Journal. We have now received feedback from two expert referees (copied below), who both consider your results on CTCF/cohesin binding and function at (sub)telomeres interesting and potentially important. While we would therefore be happy to consider a revised manuscript further for publication, there are nevertheless several substantive points that will need to be decisively clarified before eventual acceptance. In particular, both referees raise several important technical issues with the data, their controls and also their description. At the same time, the reviewers also bring up some important conceptual concerns (ref 1 point 2.2, ref 2 point 3) that will need conclusive responding, including some follow-up experimentation to allow more definitive understanding of the questions raised by referee 1.

In addition, there are also several editorial issues that I need to bring to your attention at this stage:
- please combine supplementary materials and figures into one single PDF, and check whether some figure reorganization would allow to reduce the current extensive number of supplementary figures e.g. by incorporation of some more relevant data into the main article.
- please carefully revise the reference list to conform with our citation format, but also to ensure inclusion of all relevant primary references, especially regarding TERRA function/regulation and recent works on cohesin and telomeres. Note that EMBO Journal articles have no restrictions on the number of cited primary references.
- we will need brief Conflict of Interest and Author Contribution statements at the end of the manuscript text (next to the Acknowledgements)

Please be reminded that it is our policy to allow a single round of major revision only, and that it will therefore be important to diligently and comprehensively answer to all the specific points raised at this stage in the process. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Manuscript by Deng et al.

This is the first report of localization and function of cohesin and CTCF at sub-telomere. So far there are many publications regarding genome-wide cohesin and CTCF co-localization and their function in transcriptional regulation, very little has been reported for their localization in or close to repetitive sequences. In this sense, this paper is challenging and reports very important finding but obviously additional experiments are required to justify the authors' claim.
There are two important findings made by authors
1) CTCF, Cohesin, and PolII localize at sub-telomeric region
2) Binding of CTCF is required for TERRA transcription, proper localization of TRF1/2, and telomere end protection

The first point is supported well by the current data sets but the second point is weak and the conclusion needs to be re-considered or additional experiments are required.

Major points

For point 1
1. Information brought by Point #1 is very useful, and would benefit other telomere related studies. However, for the evaluation of the results, important information is still lacking. They need at least to show ChIP-seq statistics, that summarize total sequence reads #, uniquely mapped reads#, non-uniquely mapped reads #, and reads# mapped to each class of repetitive sequence, for ChIP and WCE, respectively.
2. For the evaluation of ChIP-seq, they have performed ChIP-qPCR. This is fine, but do the primer sets they used are unique or do they expect multiple hits? As the regions they focused on include repetitive and new sequences, they should at least mention whether they amplified single band or there are possibilities of multiple bands (better to show PCR band itself or melting curve).

For point2
1. Their claim that CTCF is required for TERRA transcription in vivo is weak (Fig4F and G). How significant the differences between control and KD? p-value have to be presented.

2. According to the introduction, TERRA transcription is required for the maintenance of H3K9me3 localization. But when we look at Fig6D, it is hard to say that CTCF (and Cohesin) is required for the maintenance. Doesn't this suggest that CTCF (and Cohesin) are required for telomere end protection through other pathway than TERRA transcription? As CTCF is an insulator protein that can generally affect transcription, even when TERRA transcription is affected by KD of CTCF, isn't it hard to say that it is a direct (specific) consequence of loss of binding of CTCF at sub-tel region? On the other hand, dependencies of localization of TRF1/2 at sub-tel on CTCF/Cohesin are very clear. Are there any possibilities that CTCF regulates TRF1/2 binding through other pathways than TERRA transcription? They had better tone down or need to show more convincing evidences.

Minor point
1. Page10, line 5 and 6. They mentioned that luciferase activities are reduced to 6 fold and 2fold (FIg.4A) but from the figure I got the impression that the differences are not that big.

Referee #2 (Remarks to the Author):

In this paper, Deng et al. identify binding sites of the chromatin binding factor CTCF and cohesin subunits in the subtelomeric region near TERRA promoters. H3K4me2, H3K4me3, H3K9me2, and H3K9me3 binding to subtelomeric DNA is also characterized. Recombinant CTCF binds in electromobility shift assays (EMSA) to oligonucleotides containing encompassing the subtelomeric sequences to which CTCF binds in ChIP. TERRA promoter activity measured with luciferase reporter constructs was slightly reduced when the CTCF binding site was deleted. Knock down of CTCF expression or Rad21 (cohesion) caused a roughly 40% reduction of TERRA. At the same time RNAPII association with the subtelomere was reduced. TRF1 and TRF2 (for CTCF-KD) binding to telomeres was also reduced in the knock down experiments. It is also reported that telomere damage is increased approximately 3-fold upon CTCF or Rad21 depletion. Overall, although somewhat descriptive, the study makes very useful contributions to the understanding of chromatin composition in the subtelomeric regions of human chromosomes and the regulation of TERRA.

Critique points:

1. EMSA (Fig. 1C): The method needs to be described. I presume double stranded DNA was used but this is not obvious from the text. What were the concentrations of oligonucleotide and CTCF, and buffer composition? How long was the incubation time and did this allow reaching an equilibrium in the binding reaction? Is the apparent Kd for the here tested sequences comparable to previously characterized binding sites?

2. The reduced binding of TRF1 and TRF2 to telomeric DNA upon CTCF-depletion is shown in Fig. S11A. I find this data important and recommend including it with the main Figures. However, two shRNAs should be tested in this experiment for each factor to exclude off-target effects.

3. No telomere length changes were seen in HCT116 cells from which CTCF or Rad21 were depleted. This result seems very unexpected as depletion of TRF1 leads to strong telomere elongation in telomerase-positive cells. This apparent discrepancy should be discussed.

1st Revision - authors' response 27 July 2012

Response to Editors' comment:

While we would therefore be happy to consider a revised manuscript further for publication, there are nevertheless several substantive points that will need to be decisively clarified before eventual acceptance. In particular, both referees raise several important technical issues with the data, their controls and also their description. At the same time, the reviewers also bring up some important

conceptual concerns (ref 1 point 2.2, ref 2 point 3) that will need conclusive responding, including some follow-up experimentation to allow more definitive understanding of the questions raised by referee 1.

In the revised manuscript, we have made substantial changes to address the editor and reviewers' comments. While the point-by-point rebuttal is listed below, we provided a brief summary of these changes. In Fig. 2, we replaced the original Fig. 2D with the revised Fig. 2D, which include EMSA data of candidate CTCF binding sites from XYq, 10q and 7p subtelomeres; we also added new Fig. 2E to show the inhibition constants of these sites over a known CTCF sites in H19 imprinting control region by Fluorescence Polarization assay. The sequence information of the probes in the original Fig. 2E was listed in new Supplementary Table III. In Figs. 4 and 5, we included the statistic analysis of TERRA quantification by Northern blotting. In Fig. 6, the original Fig. S11 was incorporated as the revised Fig. 6E-G, as suggested by the reviewer and editor. In Fig. 7, we removed original model Fig. 8, and instead incorporated it into revised Fig. 7 as Fig. 7E.

In addition to the modifications in the main figures, we also included several new supplementary figures and files to address the reviewers' concerns. These include: new Fig. S1E and Supplementary File 2 to show the comparison between multi-mappings and unique mappings of ChIP-Seq datasets, and the statistics of each analysis; new Fig. S11 to eliminate potential off-target effects of shCTCF by the use of multiple shRNA against CTCF in ChIP assay; new Figs. S15 and S16 to provide the quality controls for qPCR primers of subtelomeric regions used in ChIP-qPCR and RT-PCR; revised Supplementary Table I and II to provide the information on copy number and known loci amplified by the qPCR primers. The justification for the use of these primers was included in the related Method sections in the revised manuscript.

Besides the major changes in Figures and Supplementary materials, we also revised the Result and Discussion sections to reflect these changes and to address the reviewers' concerns.

In addition, there are also several editorial issues that I need to bring to your attention at this stage:
- please combine supplementary materials and figures into one single PDF, and check whether some figure reorganization would allow to reduce the current extensive number of supplementary figures e.g. by incorporation of some more relevant data into the main article.

We appreciate the editor's suggestions. We have reorganized our figures by incorporation some of supplementary figures into main figures. We also combine supplementary materials and figures into once single PDF.

- please carefully revise the reference list to conform with our citation format, but also to ensure inclusion of all relevant primary references, especially regarding TERRA function/regulation and recent works on cohesin and telomeres. Note that EMBO Journal articles have no restrictions on the number of cited primary references.

The reference list has been revised to conform with EMBO J. citation format. In addition, we cited a few recent works on TERRA, cohesin and telomeres.

- we will need brief Conflict of Interest and Author Contribution statements at the end of the manuscript text (next to the Acknowledgements)

The Author Contribution statements and Conflict of Interest have been added.

Referee #1 (Remarks to the Author):

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For point 1
1. Information brought by Point #1 is very useful, and would benefit other telomere related studies. However, for the evaluation of the results, important information is still lacking. They need at least to show ChIP-seq statistics, that summarize total sequence reads #, uniquely mapped reads#, non-uniquely mapped reads #, and reads# mapped to each class of repetitive sequence, for ChIP and WCE, respectively.

We now provide detailed ChIP-Seq statistics as requested in the new Supplementary File 2 and unique mapping analysis in Fig. S1E. We also discuss in more detail regarding multi-mappings vs unique mappings in the Result section.

2. For the evaluation of ChIP-seq, they have performed ChIP-qPCR. This is fine, but do the primer sets they used are unique or do they expect multiple hits? As the regions they focused on include repetitive and new sequences, they should at least mention whether they amplified single band or there are possibilities of multiple bands (better to show PCR band itself or melting curve).

We thank the reviewer for this comment. We have re-examined the primer sets used for subtelomeric amplification, and noticed that a subset of these primer sets have multiple hits in other subtelomeric loci. We have included this information in the supplementary Table S2 and 3. We also show melting curves for all primer sets used for ChIP-qPCR and qRT-PCR (Supplementary Fig. S15 and S16). Despite the non-redundancy of some primer pairs to subtelomere duplications, the melting curves indicate that a single, major species is amplified for each primer set. The justification for the use of these primers was described in the Materials and Methods section.

For point2
1. Their claim that CTCF is required for TERRA transcription in vivo is weak (Fig4F and G). How significant the differences between control and KD? p-value have to be presented.

We agree with the reviewer that CTCF depletion only leads to moderate (~50%) reduction of TERRA levels, but the reduction of TERRA levels was observed consistently. We have included statistical analysis of TERRA transcription between shControl, shCTCF, and shRAD21 from Northern blotting in the revised Fig. 4F and Fig. 5D. In all cases, the p-values are considered to be significant ($P<0.05$). The moderate reduction of TERRA levels by CTCF depletion might reflect the difficulty in sustained knock-down of CTCF, as well as the complexity of TERRA transcriptional regulation.

2. According to the introduction, TERRA transcription is required for the maintenance of H3K9me3 localization. But when we look at Fig6D, it is hard to say that CTCF (and Cohesin) is required for the maintenance. Doesn't this suggest that CTCF (and Cohesin) are required for telomere end protection through other pathway than TERRA transcription? As CTCF is an insulator protein that can generally affect transcription, even when TERRA transcription is affected by KD of CTCF, isn't it hard to say that it is a direct (specific) consequence of loss of binding of CTCF at sub-tel region? On the other hand, dependencies of localization of TRF1/2 at sub-tel on CTCF/Cohesin are very clear. Are there any possibilities that CTCF regulates TRF1/2 binding through other pathways than TERRA transcription? They had better tone down or need to show more convincing evidences.

We appreciate the concerns of the reviewer, and agree that CTCF and cohesin may have other effects on telomere regulation and chromatin structure than just TERRA transcription. We have tried to include this point in the revised Discussion, and toned down the conclusion that the major effect is on TERRA transcription. The most compelling argument for CTCF having a direct role on

TERRA transcription is the strong evidence that CTCF-cohesin play a role in RNA polymerase II loading and elongation at the TERRA promoter region.

Minor point
1. Page10, line 5 and 6. They mentioned that luciferase activities are reduced to 6 fold and 2 fold (FIg.4A) but from the figure I got the impression that the differences are not that big.

We thank the reviewer for finding this error. We have corrected the numbers in the text. The corrected values are ~4 fold and ~1.5 fold.

Referee #2 (Remarks to the Author):

In this paper, Deng et al. identify binding sites of the chromatin binding factor CTCF and cohesin subunits in the subtelomeric region near TERRA promoters. H3K4me2, H3K4me3, H3K9me2, and H3K9me3 binding to subtelomeric DNA is also characterized. Recombinant CTCF binds in electromobility shift assays (EMSA) to oligonucleotides containing encompassing the subtelomeric sequences to which CTCF binds in ChIP. TERRA promoter activity measured with luciferase reporter constructs was slightly reduced when the CTCF binding site was deleted. Knock down of CTCF expression or Rad21 (cohesion) caused a roughly 40% reduction of TERRA. At the same time RNAPII association with the subtelomere was reduced. TRF1 and TRF2 (for CTCF-KD) binding to telomeres was also reduced in the knock down experiments. It is also reported that telomere damage is increased approximately 3-fold upon CTCF or Rad21 depletion. Overall, although somewhat descriptive, the study makes very useful contributions to the understanding of chromatin composition in the subtelomeric regions of human chromosomes and the regulation of TERRA.

Critique points:

1. EMSA (Fig. 1C): The method needs to be described. I presume double stranded DNA was used but this is not obvious from the text. What were the concentrations of oligonucleotide and CTCF, and buffer composition? How long was the incubation time and did this allow reaching an equilibrium in the binding reaction? Is the apparent Kd for the here tested sequences comparable to previously characterized binding sites?

The detailed methods of EMSA are now included in the revised methods section. In addition, we provided new Fig. 2E, in which these dsDNA probes with candidate CTCF sites from subtelomeres were used to compete for a labeled probe with known CTCF binding kinetics (CTCF site at mouse H19 Imprinting Control Region) by Fluorescence polarization assay. In agreement with our EMSA results, we found that only the wild type but not mutant probes show comparable dissociation constant (Ki value). In addition, we included two new authors who have helped us to calculate the Ki for these CTCF sites (Rob Plasshaert and Marissa Bartolomei).

2. The reduced binding of TRF1 and TRF2 to telomeric DNA upon CTCF-depletion is shown in Fig. S11A. I find this data important and recommend including it with the main Figures. However, two shRNAs should be tested in this experiment for each factor to exclude off-target effects.

As the reviewer suggested, we have included this data in the revised Fig. 6E-G. We also tested three shRNA for CTCF depletion in Western blotting, and used two of them in ChIP-qPCR assay in new Fig. S11. In brief, we found that both shCTCF can reduce TRF1 and TRF2 binding at subtelomere, and the level of TRF1/2 reduction correlates to the efficiency of CTCF depletion and the amount of CTCF binding at subtelomeric CTCF site.

3. No telomere length changes were seen in HCT116 cells from which CTCF or Rad21 were depleted. This result seems very unexpected as depletion of TRF1 leads to strong telomere elongation in telomerase-positive cells. This apparent discrepancy should be discussed.

Telomere length regulation by TRF1 in telomerase-positive cells occurred in a gradual and progressive manner, which required either long-term TRF1 overexpression or depletion (van Steensel B and de Lange T., Nature, 1997). In our assay in HCT116 cells, telomere length was measured at 10 days post shRNA infection, and also, TRF1 binding at telomeres was only partially

reduced. This may be the reason that we did not observe significant telomere lengthening. In addition, we could not knock down either CTCF or Rad21 for a longer period without loss of cell viability or shRNA reversion. After two weeks shRNA depletion, even under puromycin selection, we noticed that CTCF or Rad21 protein level was somehow recovered. This revertant phenotype prevents us to gain data on telomere length change by shCTCF or shRad21 in a longer period of analysis.

Acceptance letter 29 August 2012

Thank you for submitting your revised manuscript for our consideration. After some delay related to the vacation season, it has now been seen once more by the two original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal. There are only two minor points mentioned by referee 2 that may require additional textual modifications, so I would like to ask you to simply email us a modified text file containing such changes as appropriate, and we would upload this final version of the text into our system.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely,
Editor
The EMBO Journal

Referee #1

(Remarks to the Author)
I have re-reviewed the manuscript by Lieberman and coworkers. The authors have completed extensive work to address the reviewers' comments, and the manuscript provides well documented advances that in my view are of sufficient interest to be published in Embo J.

Referee #2

(Remarks to the Author)
The revised version is good. However, for the EMSA description indicate molar probe concentration and the molar concentration of CTCF. In addition, I think the indicated amount of 0.5g poly (dI-dC) cannot be correct.

